CERTIFICATE OF FACSIMILE TRANSMISSION

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Karen Zielen — Name

June 29, 2004 — Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors: Choy-Pik Chiu & Robert Kay

Filing Date: November 21, 2001

Serial No: 09/990,522

Docket: 097/002

Title: TOLERIZING ALLOGRAFTS OF PLURIPOTENT STEM CELLS

Art Unit: 1636

Examiner: Quang Nguyen, Ph.D.

DECLARATION UNDER 37 CFR § 1.132
BY JOSEPH D. GOLD, Ph.D.

Commissioner for Patents
Alexandria VA 22313

Dear Sir:

I, JOSEPH GOLD, do hereby declare as follows:

I am Associate Director of Stem Cell Biology and project leader of the Cardiovascular Disease project at Geron Corporation. I direct a team of scientists that is making cardiomyocytes from human embryonic stem (hES) cells. I also oversee ongoing projects with external collaborators for preclinical testing of the cardiomyocytes in animal models.

A copy of my *curriculum vitae* accompanies this Declaration.

This patent application describes a system we have been working on in which hES cells are differentiated into two cell populations: the first, a toleragenic cell population (exemplified by hematopoietic or mesenchymal cells); the second, a therapeutic cell population (exemplified by neurons, cardiomyocytes, islet cells, and other alternatives). The idea is that the first cell population will render the host tolerant to the histocompatibility type of the hES cell line being used. The second cell population can then be given for regenerative medicine, with reduced risk of allograft rejection.

I understand the Examiner has questioned whether cardiomyocytes can be made from hES cells as described in this application, and whether they are capable of being transplanted.

The patent application describes how to make cardiomyocytes from hES cells on pages 10-12. Our current method involves: a) culturing hES cells to form embryoid bodies in nutrient medium containing fetal calf serum; b) plating and culturing the EB cells for 2 to 3 weeks; and c) separating out beating cell clusters using Percoll®, a density gradient. This is essentially as described in this patent application. The use of 5-azacytidine in the initial derivation step (page 12 of the specification) is no longer used, and subsequent culturing of the cells in a medium containing creatine, carnitine, and taurine is optional.

Figure 1 which accompanies this Declaration is a summary of the procedure, as we presented it this June at the annual meeting of the International Society for Stem Cell Research (ISSCR).

The ISSCR presentation also showed results of transplantation experiments we conducted in conjunction with Michael A. Laflamme, Veronica Poppa, and Charles E. Murry in the Department of Pathology, University of Washington, Seattle.

The procedure was as follows. About 5.0×10^6 of hES cell derived cardiomyocytes in 70 μL medium were injected into the anterior left ventricular myocardium of anesthetized intubated male nude (Rh-rnu/rnu) rats. At various times after engraftment, animals were sacrificed, and heart tissue was fixed for paraffin-embedded for histology. The graft lineages were followed by in situ hybridization for the human Y chromosome or with a commercially available probe directed against repetitive human Alu sequences. The hESC-derived cardiac implants were also quite distinct morphologically and could be readily distinguished from the host on the basis of their exclusive

immunoreactivity for β-myosin heavy chain, versus the host ventricular myocardium which expressed only α-myosin heavy chain.

Figure 2 shows results of a time course study of hES cell derived cardiomyocyte grafts. Sarcomeric myosin heavy chain (sMHC; brown staining) at 1 day, 1 week, and 4 weeks following transplantation identifies host and graft cardiomyocytes. The three intermediate panels show immunostaining for epithelial cells (pan-cytokeratins) on adjacent sections. Over time, the tightly clustered human myocardial implants (enclosed within dotted lines) appear to have expanded; epithelial elements decrease in extent and are totally absent by 4 weeks. Human origin is confirmed by in situ hybridization with a human-specific probe against the Y-chromosome (punctuate intranuclear dots) on adjacent sections, these from fields corresponding to the insets in the above panels. (sMHC and CK images at 200X; Y in situ images magnified an additional 2.5-fold).

Figure 3 shows marker expression of the engrafted cells after 4 weeks:

Panel A: clustered human ESC-derived cardiomyocyte graft cells are positive for sarcomeric actin (red) and are identified by human-specific Y-chromosome ISH (punctuate brown intranuclear signal, best seen in inset magnified an additional 3-fold).

Panel B: distinctive vacuolated appearance of the engrafted cardiac cells by H&E staining.

Panel C: the engrafted cardiomyocytes are heavily glycogen-laden by PAS-reactivity, which is completely removed by preceding amylase digestion (**Panel D**).

Panel E: the cardiomyocyte graft cells express the ventricular-specific marker myosin light chain 2 v.

Panel F: the engrafted cardiomyocytes occasionally assume sarcomeric banding and alignment with host fibers (inset magnified an additional 2-fold). Panels A-E acquired at 400X; panel E 600X.

Table 1 shows the marker expression of engrafted cells as a function of time. Entries refer to the extent (first parameter) and intensity (second parameter) of the immunostaining for the listed antigens.

Figure 4 demonstrates that the engrafted hES cell derived cardiomyocytes are highly proliferative.

Panel A: 4 week-old cardiac implant stained for β-myosin heavy chain (red) and a human-specific antibody against the proliferative marker Ki-67 (brown intranuclear staining). Representative Ki-67 positive cardiomyocyte nuclei are indicated by the insets (magnified an additional 3-fold) and arrows.

Panel B: another 4-week old implant is stained for β-myosin heavy chain (red) and anti-BrdU (brown intranuclear staining). Two clear-cut BrdU positive and β-myosin heavy chain positive human cardiomyocytes are indicated by the arrow and rightward inset. Leftward inset: infrequent mitotic figures were noted within the human cardiomyocytes. 400X (insets magnified an additional 3-fold).

Panel C: 1 week post-transplantation 28.3 ± 5.4 % of β-myosin heavy chain positive graft cells were Ki-67 positive and 6.4 ± 0.8 % were BrdU positive. At 4 weeks following transplantation, 15.8 ± 3.4 % of graft cardiomyocytes were Ki-67 positive and 2.7% ± 0.3 % were BrdU positive.

In summary, hES cells can be differentiated into cardiomyocyte lineage cells as described in this patent application. The cells undergo spontaneous contraction, and have cell markers characteristic of human cardiomyocytes. They can be engrafted into cardiac tissue in an established preclinical model. The engrafted cells integrate into the host tissue, and undergo a process of further expansion in situ that actually enriches and expands cells having a cardiomyocyte phenotype.

We are optimistic that hES cell derived cardiomyocytes will be an important form of regenerative medicine for patients affected by heart disease. The immunotolerance strategy described in this patent application is expected to facilitate engraftment of the cardiomyocytes in human subjects.

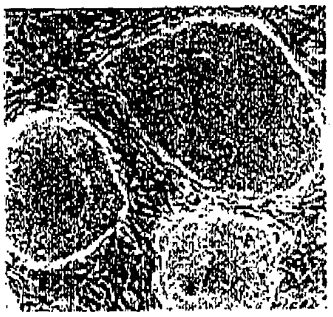
I hereby declare that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

6/25/04
Date

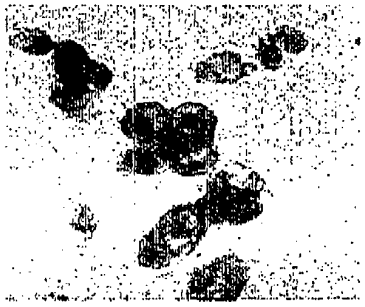
Joseph D. Gold
Joseph D. Gold, Ph.D.
Menlo Park, California

Figure 1

Cardiomyocyte isolation



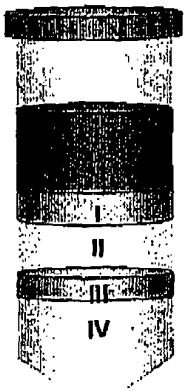
Feeder-free cultures of hES cells (H1, H7)



4d embryoid bodies in 20% FBS, 14-21 days adherent culture on gelatin

40.5% Percoll

58.5% Percoll



Fraction IV: 10% total cells, 15-20% Cardiomyocytes

Typically graft 5 x 10^5 cells into the left ventricular wall of nude rats

Table 1

Antigen	1 day	1 week	2 weeks	4 weeks
Sarcomeric actin	+/+++	++/+++	++/+++	+++/+++
β-MHC	+/+++	++/+++	++/+++	+++/+++
α-MHC	-	-	-	-
Smooth muscle α-actin	+/++	++/++	++/++	+++/+++
N-cadherin	+/+	++/+	++/++	+++/+++
Atrial natriuretic peptide	+/+	++/++	++/++	+++/+++
Myosin light chain 2v	NE	++/++	++/++	+++/+++
Connexin 43	-	-	-	-
Pan-cytokeratins	+++/+++	+/+++	+/+++	-
α-fetoprotein	+/+++	-	-	-
β III tubulin	-	-	-	-
S-100 protein	-	-	-	-
Fast skeletal myosin heavy chain	-	-	-	-

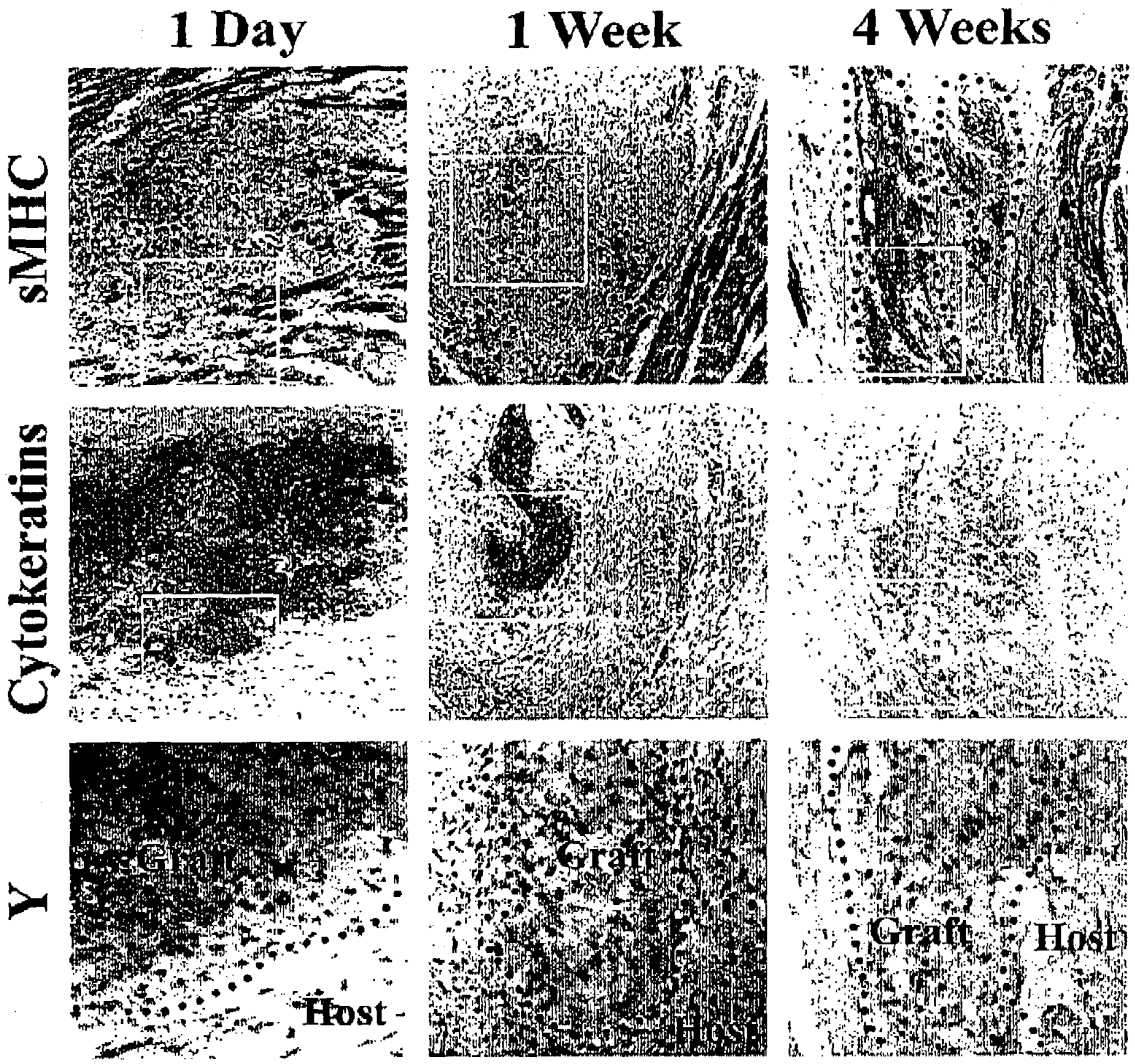

1 Day
1 Week
4 Weeks
sMHC
Cytokeratins
Y
Graft
Host
Graft
Host
Graft
Host

Figure 2

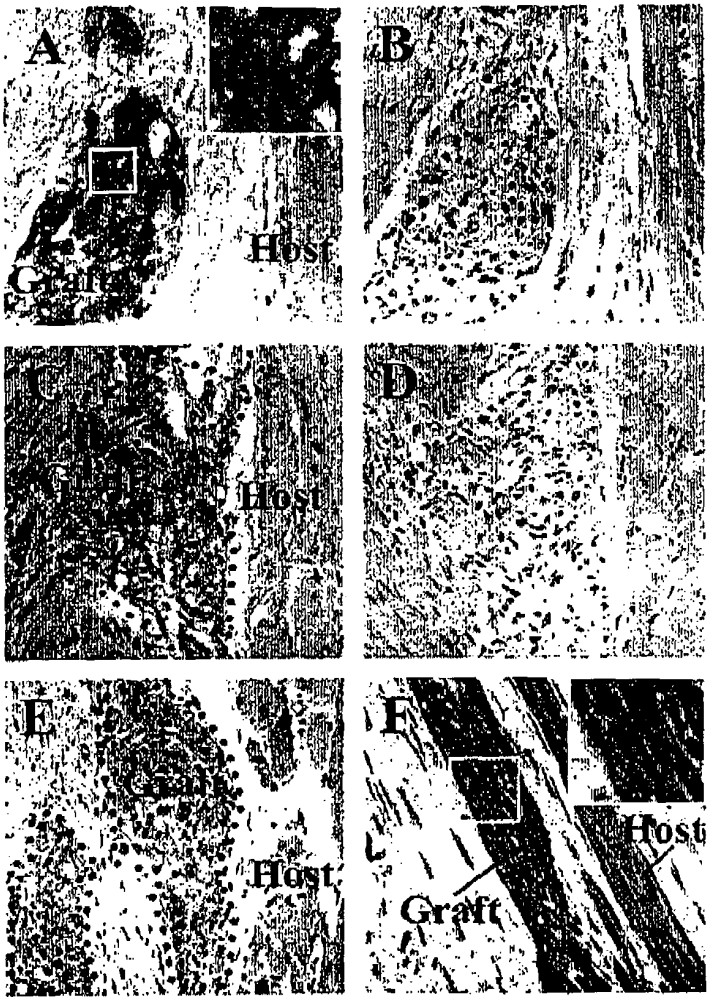


Figure 3

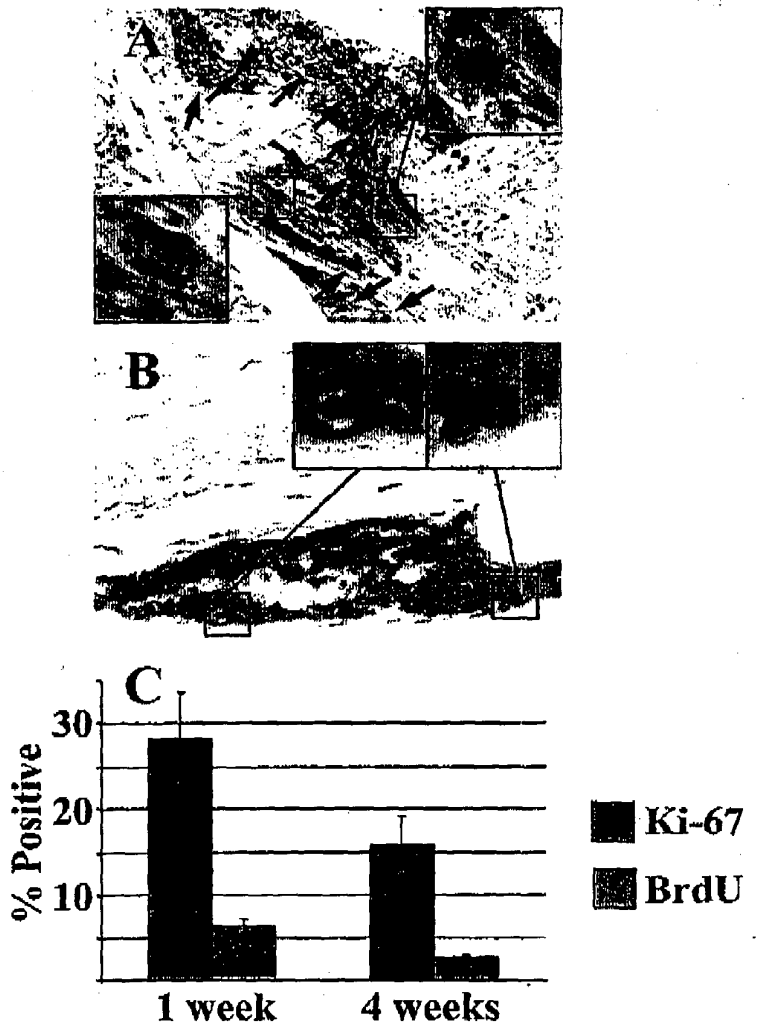


Figure 4

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Experience:

Associate Director, Stem Cell Biology
1/2003-present

Project Leader,
Cardiovascular Disease Project
6/2002-present
Direct team engaged in generation of cardiomyocytes from human ES cells using growth factor, transduction, and other cell-culture mediated approaches, coordination of in house efforts and external transplantation programs, manage external collaborations

Group Leader, Genetic Engineering, Geron Corporation
1/2001-present
Genetic modification of human ES cells, optimization of plasmid and viral vectors; generation of transiently and stably modified cell lines designed to influence differentiation, minimize transplant safety concerns, avoid immunological rejection

Team Leader, Embryonic Stem Cell Program, Geron Corporation
7/98-6/2002
Responsible for managing nonhuman and human ES cell program; examining gene expression in undifferentiated and differentiated ES cells; identification and validation of markers for characterization of human ES cells, transfection/transduction studies; coordinating and integrating efforts of Cell Biology, Molecular Biology, Assay Development and Vector Biology groups.

Staff Scientist III, Geron Corporation, 230 Constitution Drive, Menlo Park, CA 94025
7/98

Staff Scientist II, Geron Corporation, 230 Constitution Drive, Menlo Park, CA 94025 6/97

Staff Scientist I, Geron Corporation, 230 Constitution Drive, Menlo Park, CA 94025 6/97
1/96-5/97
Initiated culture of primate ES cells; optimized primate ES cell medium; conducted medium throughput screen of growth factors, cytokines, extracellular matrix components and small molecules for effects on ES cell growth

Postdoctoral Fellow, Laboratory of Dr. Roger Pedersen, Laboratory of Radiobiology and Environmental Health, University of California at San Francisco, San Francisco, California 10/90 to 12/95
Investigated the role of genes involved in embryonic development in the mouse by molecular biology: overexpressed genes in ES cells and studied the results both in cell culture and in transgenic mice; constructed subtracted cDNA libraries from transfected ES cells and cloned novel induced genes; examined the role of developmental genes through homologous recombination m ES cells and evaluated the results both in cell culture and in transgenic mice

Graduate student, Laboratory of Dr. Lorraine Gudas, Dana Farber Cancer Institute, Harvard University, Boston, Massachusetts. 6/84 to 10/90
Studied mechanism of retinoid action in F9 teratocarcinoma cells; identified and characterized a retinoic acid responsive element in the promoter of the murine laminin B1 gene; overexpressed and characterized retinoic acid receptors in COS cells and analyzed interactions between receptors and DNA in vivo and in vitro

Undergraduate student, Laboratory of Dr. Andrew Binns, University of Pennsylvania Philadelphia, Pennsylvania 9/82 to 9/83
Studied meiotic transmission of T-DNA in tobacco plants

Publications:

Formation of human myocardium in the rat heart from human embryonic stem cells. Michael A. Laflamme, Joseph Gold, Chunhui Xu, Mohammad Hassanipour, Elen Rosler, Shailija Police, Veronica Poppa, and Charles E. Murry
Submitted

Differentiated human embryonic stem cells survive in vivo and improve cardiac function following injection into injured mouse myocardium. Theo Kofidis, Jorg L. Debruin, Rutger-Jan Swijnenburg, Jonathan Hardy, Timothy C. Doyle, Christopher Contaq, Chunhui Xu, Joseph Gold, Gerry Berryy, and Robert C. Robbins.
Submitted

Basic fibroblast growth factor supports undifferentiated human embryonic stem cell growth without conditioned medium. Chunhui Xu, Elen Rosler, Jianjie Jiang, Jane S. Lebkowski, Joseph D. Gold, Chris O'Sullivan, Karen Delavan-Boorsma, Michael Mok, Adrienne Bronstein and Melissa K. Carpenter
Submitted

Identification of marker genes expressed on undifferentiated human embryonic stem cells by a genomics-based strategy. Lawrence W. Stanton, Yan Li, Michael Mok, Elisa Brunette, Ralph Brandenberger, John Irving, Joseph Gold, and Ramkumar Mandalam
In preparation

Lebkowski JS, Gold J, Xu C, Funk W, Chiu CP, Carpenter MK. Human embryonic stem cells: culture, differentiation, and genetic modification for regenerative medicine applications.
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Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK Feeder-free growth of undifferentiated human embryonic stem cells.
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Boucher DM, Schaffer M, Deissler K, Moore CA, Gold JD, Burdsal CA, Meneses JJ, Pedersen RA, Blum M. *goosecoid* expression represses Brachyury in embryonic stem cells and affects craniofacial development in chimeric mice.
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Villar, A.J., J.D. Gold, K.E. Mate, J.J. Meneses, K.J. McLaughlin and R.A. Pedersen. Both parental alleles of imprinted genes are expressed in mouse blastocysts and embryonic stem cells. Submitted.

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Vasios, G.W., J.D. Gold, M. Petkovich, P. Chambon and L.J. Gudas. A retinoic acid responsive element is present in the 5' flanking region of the laminin B1 gene. *Proc. Natl. Acad. Sci. USA* **86**:9099-9103 (1989).

Patents and Patent Applications

US Patent 6,667,176 12/23/03 cDNA libraries reflecting gene expression during growth and differentiation of human pluripotent stem cells

US Patent 6,642,048 06/10/03 Conditioned media for propagating human pluripotent stem cells

US Patent 6,576,464 06/10/03 Methods for providing differentiated stem cells

US 2003/0224411 A1 Genes that are up- or down-regulated during differentiation of human embryonic stem cells

US 2003/0017589 A1 Culture system for rapid expansion of human embryonic stem cells

US 2002/0072117 A1 Human feeder cells that support proliferation of undifferentiated pluripotent stem cells

WO 02/14469 02/21/2002 Reprogramming Cells for Enhanced Differentiation Capacity using Pluripotent Stem Cells

AU0729377B2 02/01/2001 METHODS AND MATERIALS FOR THE GROWTH OF PRIMATE-DERIVED PRIMORDIAL STEM CELLS IN FEEDER-FREE CULTURE

WO0151616A2 07/19/2001 TECHNIQUES FOR GROWTH AND DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS

WO9960371A2 11/25/1999 METHOD AND APPARATUS FOR DETECTING HAZARDOUS AGENTS

WO9920741A1 04/29/1999 METHODS AND MATERIALS FOR THE GROWTH OF PRIMATE-DERIVED PRIMORDIAL STEM CELLS

Education:

Bachelor of Arts, University of Pennsylvania,
Philadelphia, Pennsylvania, 1983

Doctor of Philosophy, Harvard University
Cambridge, Massachusetts, 1991
Field: Cell and Developmental Biology
Graduate Advisor: Dr. Lorraine Gudas
Thesis: An Examination of the Retinoic Acid
Responsiveness of the Murine Laminin B1
Promoter

Honors:

National Merit Semi-finalist
American Legion Award
Phi Beta Kappa Society, University of Pennsylvania
Summa Cum Laude, University of Pennsylvania